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(54) **METHODS AND TARGETS OF ANTIBIOTIC RESISTANCE**

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(58) **Field of Search** 435/29, 6, 440, 435/441

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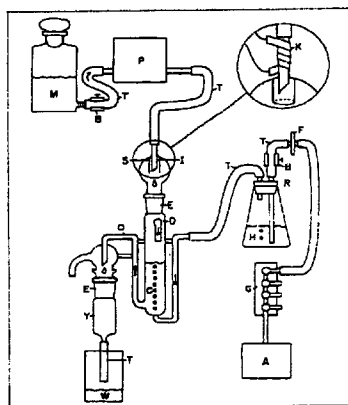
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(57) **ABSTRACT**

The present invention provides for a method of evolving and selecting cells resistant to a selective agent by inducing directed evolution in continuous culture while applying both a mutagenic and selective agent to the cells to determine the cells having resistance. This also provides a method of generating mutant drug targets useful for screening for effective compounds. The Figure is a generalized schematic diagram of a chemostat of the present invention

19 Claims, 3 Drawing Sheets



A, air pump
C, growing culture
D, air exit port
F, filter
G, gang valve
H, water humidification flask
I, input chamber
K, heating tape
M, fresh medium
O, siphon
P, peristaltic pump
W, waste

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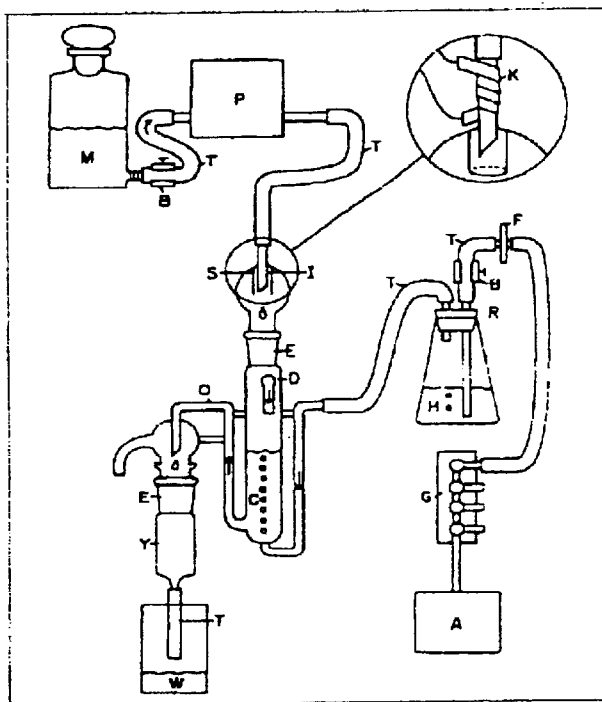
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- A, air pump
- C, growing culture
- D, air exit port
- F, filter
- G, gang valve
- H, water humidification flask
- I, input chamber
- K, heating tape
- M, fresh medium
- O, siphon
- P, peristaltic pump
- W, waste

FIGURE 1A

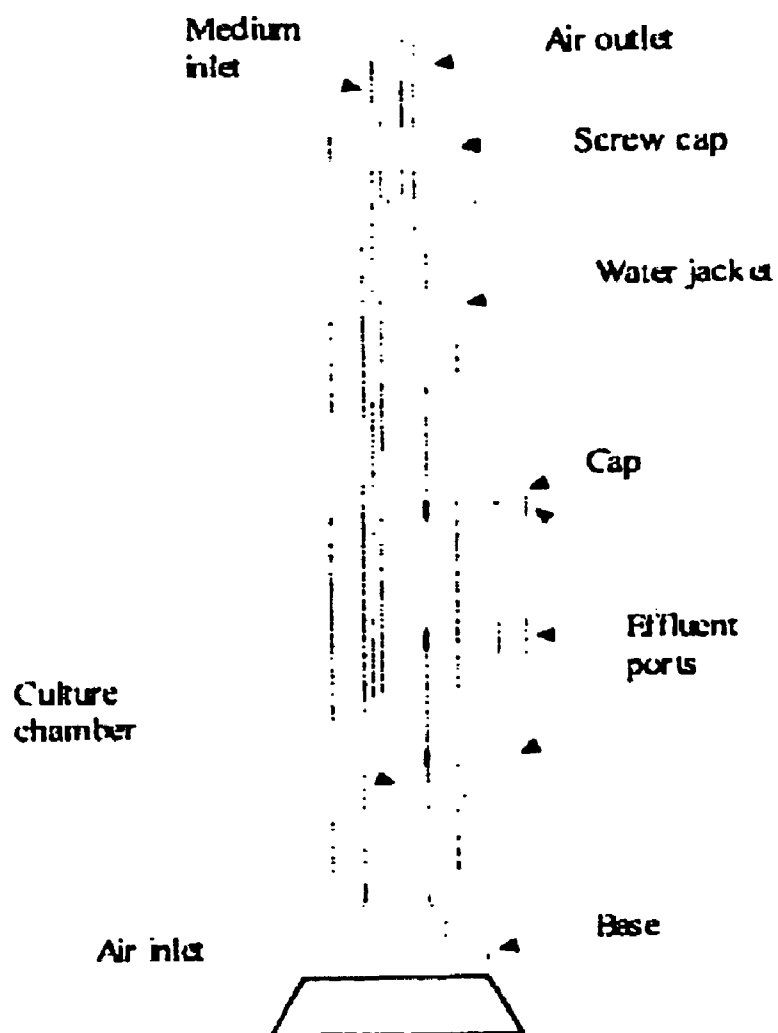


FIGURE 1B

Figure 2
Directed Evolution of the Quinolone-Resistance Determinant Region
(QRDR) of the *gyrA* Gene Sequence of *E. coli**

	G ⁸¹	D ⁸¹	S ⁸³	D ⁸⁷
E. coli 11775(WT)	TTGGTGACGTAATCGGTAAATACCATCCCATGGTGAATCGTCGGTTTATGCACAGCAACGTCCTGATGGCGACGCCATTCTCGCTGGTTACATGCTGGTAGACGGTC			
NX-E09	TTGGTGACGTAATCGGTAAATACCATCCCATGGTGAATCGTCGGTTTATGCACAGCAACGTCCTGATGGCGACGCCATTCTCGCTGGTTACATGCTGGTAGACGGTC			
NX-E10	TTGGTGACGTAATCGGTAAATACCATCCCATGGTGAATCGTCGGTTTATGCACAGCAACGTCCTGATGGCGACGCCATTCTCGCTGGTTACATGCTGGTAGACGGTC			
NX-M6	TTGGTGACGTAATCGGTAAATACCATCCCATGGTGAATCGTCGGTTTATGCACAGCAACGTCCTGATGGCGACGCCATTCTCGCTGGTTACATGCTGGTAGACGGTC			
NX-M3	TTGGTGACGTAATCGGTAAATACCATCCCATGGTGAATCGTCGGTTTATGCACAGCAACGTCCTGATGGCGACGCCATTCTCGCTGGTTACATGCTGGTAGACGGTC			
NX-M5	TTGGTGACGTAATCGGTAAATACCATCCCATGGTGAATCGTCGGTTTATGCACAGCAACGTCCTGATGGCGACGCCATTCTCGCTGGTTACATGCTGGTAGACGGTC			
NX-M6	TTGGTGACGTAATCGGTAAATACCATCCCATGGTGAATCGTCGGTTTATGCACAGCAACGTCCTGATGGCGACGCCATTCTCGCTGGTTACATGCTGGTAGACGGTC			
D7NX-E1	TTGGTGACGTAATCGGTAAATACCATCCCATGGTGAATCGTCGGTTTATGCACAGCAACGTCCTGATGGCGACGCCATTCTCGCTGGTTACATGCTGGTAGACGGTC			
D7NX-E2	TTGGTGACGTAATCGGTAAATACCATCCCATGGTGAATCGTCGGTTTATGCACAGCAACGTCCTGATGGCGACGCCATTCTCGCTGGTTACATGCTGGTAGACGGTC			
D3NX-E5	TTGGTGACGTAATCGGTAAATACCATCCCATGGTGAATCGTCGGTTTATGCACAGCAACGTCCTGATGGCGACGCCATTCTCGCTGGTTACATGCTGGTAGACGGTC			
D3NX-E6	TTGGTGACGTAATCGGTAAATACCATCCCATGGTGAATCGTCGGTTTATGCACAGCAACGTCCTGATGGCGACGCCATTCTCGCTGGTTACATGCTGGTAGACGGTC			
D2C2-a	TTGGTGACGTAATCGGTAAATACCATCCCATGGTGAATCGTCGGTTTATGCACAGCAACGTCCTGATGGCGACGCCATTCTCGCTGGTTACATGCTGGTAGACGGTC			
D2C2-h	TTGGTGACGTAATCGGTAAATACCATCCCATGGTGAATCGTCGGTTTATGCACAGCAACGTCCTGATGGCGACGCCATTCTCGCTGGTTACATGCTGGTAGACGGTC			
D2C2-i	TTGGTGACGTAATCGGTAAATACCATCCCATGGTGAATCGTCGGTTTATGCACAGCAACGTCCTGATGGCGACGCCATTCTCGCTGGTTACATGCTGGTAGACGGTC			

* G = Glycine, D = Aspartic Acid, S = serine (amino acid positions are numbered as superscripts).

Single or double base pair point mutations are shown in blue letters and the mis-matching bases are shown in red letters.

METHODS AND TARGETS OF ANTIBIOTIC RESISTANCE

CROSS REFERENCE TO RELATED APPLICATIONS

The present application is a National Phase Concerning a Filing Under 35 U.S.C. 371, claiming the benefit of priority of PCT/US00/40676, filed Aug. 18, 2000, which claims the benefit of priority of U.S. Provisional Ser. No. 60/149,761, filed Aug. 19, 1999 both of which are incorporated herein by reference.

BACKGROUND OF THE INVENTION

1. Field of the Invention

Generally, the present invention relates to methods and materials for the discovery and characterization of molecular mechanisms of drug resistance. More specifically, the present invention relates to methods and materials for the discovery and characterization of molecular mechanisms of drug resistance using directed evolution.

2. Description of Related Art

Drug resistance, especially that of anti-infective and anti-cancer drugs, is an extremely important aspect of the clinical use and efficacy of therapeutic compounds in the treatment of human and animal diseases. Drug resistance necessitates affirmative treatments which can be less effective and more costly. Moreover, the rapidly increasing rate of reemergence of once controlled clinical infections is seriously eroding the repertoire of effective antibiotics. For example, many common pediatric antibiotics, such as amoxicillin, have been rendered much less effective against such common infections as otitis media due to emerging resistance over the past decade. Alternative therapeutics, when available, are often much more expensive than the first line drug. Likewise, tumor cells of patients undergoing cancer treatment often become resistant to the anticancer drugs being used.

A key in determining the cause of resistance is often found in an analysis of the drug target proteins of the pathogenic organism. Mutation of the drug target protein is responsible for much of the rapidly increasing rate of clinical antibiotic resistance. This is attributable to the large number and variety of antibiotics that target cellular proteins. Likewise, changes in the target protein structure, as a result of point mutations in the gene, results in loss of binding and efficacy of the antibiotic. This has been widely observed in clinical isolates for nearly all antibiotics targeting cellular proteins.

The amino acid sequence of a protein determines its three-dimensional (3D) structure, which in turn determines protein function (EPST63, ANFI73). Shortle (SHOR85), Sauer and colleagues (PAKU86, REID88a), and Caruthers and colleagues (EISE85) have shown that some residues on the polypeptide chain are more important than others in determining the 3D structure of a protein. The 3D structure is essentially unaffected by the identity of the amino acids at some loci, while at other loci, only one or a few types of amino-acid is allowed. In most cases, the loci is where wide variety is allowed have the amino acid side-chain group directed toward the solvent. Loci where limited variety is allowed frequently have the sidechain group directed toward other parts of the protein. Thus substitutions of amino acids that are exposed to solvent are less likely to affect the 3D structure than are substitutions at internal loci. (See also SCHU79, p169-171 and CREI84, p239-245, 314-315).

The secondary structure (helices, sheets, turns, loops) of a protein is determined mostly by local sequence. Certain

amino acids have a propensity to appear in certain "secondary structures," they are found from time to time in other structures, and studies of pentapeptide sequences found in different proteins have shown that their conformation varies considerably from one occurrence to the next (KABS84, ARGO87). As a result, a priori design of proteins to have a particular 3D structure is difficult.

Several researchers have designed and synthesized proteins de novo (MOSE83, MOSE87, ERIC86). These designed proteins are small and most have been synthesized in vitro as polypeptides rather than genetically. Hecht et al. (HECH90) have produced a designed protein genetically. Moser, et al. state that design of biologically active proteins is currently impossible.

Many proteins bind non-covalently but very tightly and specifically to some other characteristic molecules (SCHU79, CREI84). In each case, the binding results from complementarity of the surfaces that come into contact: bumps fit into holes, unlike charges come together, dipoles align, and hydrophobic atoms contact other hydrophobic atoms. Although bulk water is excluded, individual water molecules are frequently found filling space in intermolecular interfaces, these waters usually form hydrogen bonds to one or more atoms of the protein or to other bound water. Thus proteins found in nature have not attained, nor do they require, perfect complementarity to bind tightly and specifically to their substrates. Only in rare cases is there essentially perfect complementarity; then the binding is extremely tight (as for example, avidin binding to biotin).

"Protein engineering" is the art of manipulating the sequence of a protein in order to alter its binding characteristics. The factors affecting protein binding are known, (CHOT75, CHOT76, SCHU79, p98-107, and CREI84, Ch8), but designing new complementary surfaces has proved difficult. Although some rules have been developed for substituting side groups (SUTC87b), the side groups of proteins are floppy (i.e. can move from side to side) and it is difficult to predict what conformation a new side group will take. Further, the forces that bind proteins to other molecules are all relatively weak and it is difficult to predict the effects of these forces.

Recently, Quioco and collaborators (QUIO87) elucidated the structures of several periplasmic binding proteins from Gram-negative bacteria. They found that the proteins, despite having low sequence homology and differences in structural detail, have certain important structural similarities. Based on their investigations of these binding proteins, Quioco et al. suggest it is unlikely that, using current protein engineering methods, proteins can be constructed with binding properties superior to those of proteins that occur naturally.

Nonetheless, there have been some isolated successes. Wilkinson et al. (WILK84) reported that a mutant of the tyrosyl tRNA synthetase of *Bacillus stearothermophilus* with the mutation Thr₅₁→Pro exhibits a 100-fold increase in affinity for ATP Tan and Kaiser (TANK77) and Tschesche et al. (TSCH87) showed that changing a single amino acid in mini-protein greatly reduces its binding to trypsin, but that some of the mutants retained the parental characteristic of binding to an inhibiting chymotrypsin, while others exhibited new binding to elastase. Caruthers and others (EISE85) have shown that changes of single amino acids on the surface of the lambda Cro repressor greatly reduce its affinity for the natural operator O_R 3, but greatly increase the binding of the mutant protein to a mutant operator. Changing three residues in subtilisin from *Bacillus amyloliquefaciens*

to be the same as the corresponding residues in subtilisin from *B. licheniformis* produced a protease having nearly the same activity as the latter subtilisin, even though 82 amino acid sequence differences remained (WELL87a). Insertion of DNA encoding 1B amino acids (corresponding to Pro-Glu-Dynorphin-Gly) into the *E. coli* *phoA* gene so that the additional amino acids appeared within a loop of the alkaline phosphatase protein resulted in a chimeric protein having both *phoA* and dynorphin activity (FREI90). Thus, changing the surface of a binding protein can alter its specificity without abolishing binding activity.

Early techniques of mutating proteins involved manipulations at the amino acid sequence level. In the semi-synthetic method (TSCH87), the protein was cleaved into two fragments, a residue removed from the new end of one fragment, the substitute residue added on in its place, and the modified fragment joined with the other, original fragment. Alternatively, the mutant protein could be synthesized in its entirety (TANK77).

Erickson et al. suggested that mixed amino acid reagents could be used to produce a family of sequencerelated proteins which could then be screened by affinity chromatography (ERIC86). They envision successive rounds of mixed synthesis of variant proteins and purification by specific binding. They do not discuss how residues should be chosen for variation. Because proteins cannot be amplified, the researchers must sequence the recovered protein to learn which substitutions improve binding. The researchers must limit the level of diversity so that each variety of protein is present in sufficient quantity for the isolated fraction to be sequenced.

With the development of recombinant DNA techniques, it became possible to obtain a mutant protein by mutating the gene encoding the native protein and then expressing the mutated gene. Several mutagenesis strategies are known. One, "protein surgery" (DILL87), involves the introduction of one or more predetermined mutations within the gene of choice. A single polypeptide of completely predetermined sequence is expressed, and its binding characteristics are evaluated.

At the other extreme is random mutagenesis by means of relatively nonspecific mutagens such as radiation and various chemical agents. See Ho et al. (HOCJ85) and Lehtovaara, E. P. Appin. 285,123.

It is possible to randomly vary predetermined nucleotides using a mixture of bases in the appropriate cycles of a nucleic acid synthesis procedure. The proportion of bases in the mixture, for each position of a codon, determines the frequency at which each amino acid occurs in the polypeptides expressed from the degenerate DNA population. Oliphant et al. (OLIP86) and Oliphant and Struhl (OLIP87) have demonstrated ligation and cloning of highly degenerate oligonucleotides, which were used in the mutation of promoters. They suggested that similar methods could be used in the variation of protein coding regions. They do not say how one should: a) choose protein residues to vary, or b) select or screen mutants with desirable properties. Reidhaar-Olson and Sauer (REID88a) have used synthetic degenerate oligonucleotides to vary simultaneously two or three residues through all twenty amino acids. See also Vershon et al. (VERS86a; VERS86b). Reidhaar-Olson and Sauer do not discuss the limits on how many residues could be varied at once nor do they mention the problem of unequal abundance of DNA encoding different amino acids. They looked for proteins that either had wild-type dimerization or that did not dimerize. They did not seek proteins having novel

binding properties and did not find any. This approach is likewise limited by the number of colonies that can be examined (ROBE86).

To the extent that this prior work assumes that it is desirable to adjust the level of mutation so that there is one mutation per protein, many desirable protein alterations require multiple amino acid substitutions and thus are not accessible through single base changes or even through all possible amino acid substitutions at any one residue.

Ferenci and collaborators have published a series of papers on the chromatographic isolation of mutants of the maltose-transport protein LamB of *E. coli* (FERE82a, FERE82b, FERE83, FERE84, CLUN84, HEIN87 and is papers cited therein). The mutants were either spontaneous or induced with nonspecific chemical mutagens. Levels of mutagenesis were picked to provide single point mutations or single insertions of two residues. No multiple mutations were sought or found.

While variation was seen in the degree of affinity for the conventional LamB substrates maltose and starch, there was no selection for affinity to a target molecule not bound at all by native LamB, and no multiple mutations were sought or found. FERE84 speculated that the affinity chromatographic selection technique could be adapted to development of similar mutants of other "important bacterial surface located enzymes", and to selecting for mutations which result in the relocation of an intracellular bacterial protein to the cell surface. Ferenci's mutant surface proteins would not, however, have been chimeras of a bacterial surface protein and an exogenous or heterologous binding domain.

Ferenci also taught that there was no need to clone the structural gene, or to know the protein structure, active site, or sequence.

Ferenci did not limit the mutations to particular loci or particular substitutions. Ferenci does not suggest that surface residues should be preferentially varied. In consequence, Ferenci's selection system is much less efficient than that disclosed herein.

A number of researchers have directed unmutated foreign antigenic epitopes to the surface of bacteria or phage, fused to a native bacterial or phage surface protein, and demonstrated that the epitopes were recognized by antibodies. Thus, Charbit, et al. (CHAR86) genetically inserted the C3 epitope of the VP1 coat protein of poliovirus into the LamB outer membrane protein of *E. coli*, and determined immunologically that the C3 epitope was exposed on the bacterial cell surface. Charbit, et al. (CHAR87) likewise produced chimeras of LamB and the A (or B) epitopes of the preS2 region of hepatitis B virus.

A chimeric LacZ/OmpB protein has been expressed in *E. coli* and is, depending on the fusion, directed to either the outer membrane or the periplasm (SILH77). A chimeric LacZ/OmpA surface protein has also been expressed and displayed on the surface of *E. coli* cells (Weinstock et al., WEIN83). Others have expressed and displayed on the surface of a cell chimeras of other bacterial surface proteins, such as *E. coli* type 1 fimbriae (Hedegaard and Klemm (HEDE89)) and *Bacterioides nodusus* type 1 fimbriae (Jennings et al., JENN89). In none of the recited cases was the inserted genetic material mutagenized.

Dulbecco (DULB86) suggests a procedure for incorporating a foreign antigenic epitope into a viral surface protein so that the expressed chimeric protein is displayed on the surface of the virus in a manner such that the foreign epitope is accessible to antibody. In 1985 Smith (SMIT85) reported inserting a nonfunctional segment of the EcoRI endonu-

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clease gene into gene III of bacteriophage fl, "in phase". The gene III protein is a minor coat protein necessary for infectivity. Smith demonstrated that the recombinant phage were adsorbed by immobilized antibody raised against the EcoRI endonuclease, and could be eluted with acid. De la Cruz et al (DELA88) have expressed a fragment of the repeat region of the circumsporozoite protein from *Plasmodium falciparum* on the surface of M13 as an insert in the gene III protein. They showed that the recombinant phage were both antigenic and immunogenic in rabbits, and that such recombinant phage could be used for B epitope mapping. The researchers suggest that similar recombinant phage could be used for T epitope mapping and for vaccine development.

McCafferty et al. (MCCA90) expressed a fusion of an Fv fragment of an antibody to the N-terminal of the pill protein. The Fv fragment was not mutated. F. Epitope Libraries on Fusion Phage

Parmley and Smith (PARM88) suggested that an epitope library that exhibits all possible hexapeptides could be constructed and used to isolate epitopes that bind to antibodies. In discussing the epitope library, the authors did not suggest that it was desirable to balance the representation of different amino acids. Nor did they teach that the insert should encode a complete domain of the exogenous protein. Epitopes are considered to be unstructured peptides as opposed to structured proteins.

Another problem with the Scott and Smith, Cwirla et al., and Devlin et al., libraries was that they provided a highly biased sampling of the possible amino acids at each position. Their primary concern in designing the degenerate oligonucleotide encoding their variable region was to ensure that all twenty amino acids were encodable at each position; a secondary consideration was minimizing the frequency of occurrence of stop signals. Consequently, Scott and Smith and Cwirla et al. employed NNK (N=equal mixture of G, A, T, C; K=equal mixture of G and T) while Devlin et al. used NNS (S=equal mixture of G and C). There was no attempt to minimize the frequency ratio of most favored to least favored amino acid, or to equalize the rate of occurrence of acidic and basic amino acids.

Devlin et al. characterized several affinity-selected streptavidin-binding peptides, but did not measure the affinity constants for these peptides. Cwirla et al. did determine the affinity constant for his peptides, but were disappointed to find that his best hexapeptides had affinities (350–300 nM), "orders of magnitude" weaker than that of the native Met-enkephalin epitope (7 nM) recognized by the target antibody. Cwirla et al. speculated that phage bearing peptides with higher affinities remained bound under acidic elution, possibly because of multivalent interactions between phage (carrying about 4 copies of pill) and the divalent target IgG. Scott and Smith were able to find peptides whose affinity for the target antibody (A2) was comparable to that of the reference myohemerythrin epitope (50 nM). However, Scott and Smith likewise expressed concern that some high-affinity peptides were lost, possibly through irreversible binding of fusion phage to target. G. Non-Commonly Owned Patents and Applications Naming Robert Ladner as an Inventor.

Ladner, U.S. Pat. No. entitled, "Computer Based System and Method for Determining and Displaying Possible Chemical Structures for Converting Double or Multiple-Chain Polypeptides to Single-Chain Polypeptides" describes a design method for converting proteins composed of two or more chains into proteins of fewer polypeptide chains, but

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with essentially the same 3D structure. There is no mention of variegated DNA and no genetic selection. Ladner and Bird, WO88101649 (Publ. Mar. 10, 1988) disclose the specific application of computerized design of linker peptides to the preparation of single chain antibodies.

Ladner, Glick, and Bird, WO88/06630 (publ. 7 Sep. 1988 and having priority from U.S. application Ser. No. 07/021, 046, assigned to Genex Corp.) (LGB) speculate that diverse single chain antibody domains (SCAD) can be screened for binding to a particular antigen by varying the DNA encoding the combining determining regions of a single chain antibody, subcloning the SCAD gene into the gpV gene of phage lambda so that a SCAD/gpV chimera is displayed on the outer surface of phage lambda, and selecting phage which bind to the antigen through affinity chromatography. The only antigen mentioned is bovine growth hormone. No other binding molecules, targets, carrier organisms, or outer surface proteins are discussed. Nor is there any mention of the method or degree of mutagenesis. Furthermore, there is no teaching as to the exact structure of the fusion nor of how to identify a successful fusion or how to proceed if the SCAD is not displayed.

Additionally, other prior art does not disclose any correlation between any of these mutations and any cellular activity. However, it has been found that the sites of antibiotic activity within target microorganisms are generally defined, and also give rise to resistance through adaptive mutations that confer resistance. Clonal propagation can also account for the spread of resistant infections in addition to independently emerging mutants.

Specifically, it would be useful to establish a correlation between these mutations and cellular activity. Also, useful is a method which shows how to implement the principles of directed evolution to evolve and discover drug resistance mechanisms and resistance conferring molecules from a presently susceptible microorganism. This would allow for predicting the time of clinical efficacy of a drug prior to its wide-spread clinical use, and the discovery and characterization of the molecule that eventually confers resistance to the target microorganism. This resistance conferring molecule would also be useful for drug screening for subsequent generation of agents for future clinical use.

SUMMARY OF THE INVENTION

According to the present invention, there is provided a method of evolving and selecting cells resistant to a selective agent by inducing directed evolution in continuous culture while applying both a magnetic and selective agent to the cells to determine the cells having resistance. This also provides a method of generating mutant drug targets useful for screening for effective compounds.

DESCRIPTION OF THE DRAWINGS

Other advantages of the present invention are readily appreciated as the same becomes better understood by reference to the following detailed description when considered in connection with the accompanying drawings:

FIG. 1A is a generalized schematic diagram of a chemostat of the present invention;

FIG. 1B is a schematic diagram of a specific chemostat of the present invention; and

FIG. 2 is a sequence alignment of the quinolone resistance determining region from resistant mutants generated with the present invention compared to the wild type.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to a method for evolving and/or selecting for microorganisms from a susceptible

culture or sample, that are resistant to one or more therapeutic agents. This agent is typically an antibiotic or anti-cancer compound. The method relies upon the principles of directed evolution and is specifically implemented through continuous culture in the presence of both selective agent (antibiotic) and mutagen.

A "chemostat" as used herein is meant to include any apparatus which properly controls the environment such that bacterial culture is maintained in a continuous state of cell division. Any chemostat can be used that is appropriate for the experimental conditions at hand as are known to those of skill in the art. A generalized schematic is shown in FIG. 1A (Dykhuizen, 1993), and can be of simple, ready-made and/or of custom design. A proprietary design for a very versatile, variable volume chemostat is shown in FIG. 1B.

The term "selective agent" as used herein is meant to include any chemotherapeutics or any other compound that can act to permit phenotypic or genotypic differentiation between mutant and wild type cells. Additional examples would include industrial chemicals that can also be used as nutrients by microorganisms. Mutagens used in the present invention include Ethyl Methanesulfonate (EMS), 4Nitroquinoline-1xide (NQO), and N-Methyl-N'-Nitro-N-Nitrosoguanidine (MNNG), but can include any magnetic agent, physical or chemical, without departing from the disclosure of the present invention. For example, UV irradiation of a chemostatic culture, so specially constructed of UV transparent glass can be implemented. The design of the chemostat shown in FIG. 1B is especially amenable to this application due to the columnar shape and relatively light transparent culture vessel with the chemostat. However, any apparatus that can provide for continuous culture, including chemostats, nutristats, and fermentors, can be is used in the present invention.

In the practice of the present invention, the drug sensitive microorganism is cultured in a chemostat via continuous culture. The selective agent (e.g. antibiotic) is also added to the culture medium either continuously or in a stepwise manner. Additionally, mutagenic agents are added to the continuous culture at a concentration predetermined by a survivability vs. dose curve. The optimum concentration of mutagen produces the maximum number of non-lethal mutations.

The optimum concentration of mutagen is determined empirically by determining a survivability vs. dose curve. This concentration must be determined empirically for each pure strain or mixed culture used. In general, for stepwise mutagenesis and selection, the optimum concentration for many bacteria, including several strains of *E. coli*, is approximately 0.5–3% (v/v or w/w) for 20 to 120 minutes. Alternatively, the mutagen can be infused slowly and continuously into the continuous culture, with adjustments, if necessary, in reduced medium influx to compensate for decreasing numbers of cells in culture as a result of lethal mutations. This is also determined empirically from the survivability vs. dose curve.

A typical evolution/discovery chemostat consists of a 100 to 1000 ml culture grown for several generations to establish stability and equilibrium. Subsequently, mutagen is added to the chemostatic culture, as described in the preceding paragraph, to the predetermined concentration of mutagenic agent. Subsequently, antibiotic selection is commenced at a predetermined concentration (either step-wise or constant infusion). Typically, and as in the case of antibiotic used for selection, the selective agent concentration is titrated to give a differential growth rate between wild type and resistant mutants.

While the presence of a selective agent in the culture provides an ongoing estimate of increasing resistance, a more detailed analysis is provided by submitting a small portion of the culture to an antibiotic susceptibility test, such as a simple antibiotic gradient plate, at various times during directed evolution. Analyzing all the resistant colonies allows for the entire evolutionary spectrum of resistance to be determined. Control chemostats, lacking mutagen, are also monitored in this manner in order to differentiate emerging resistance caused by mutagenesis from clonal selection of preexisting variants (mutants). However, in either case, the present invention provides similar benefits in providing the isolation and characterization of resistant mutants, not otherwise available.

In an embodiment of the present invention, the use of a material that mimics a physiological attachment or colonization site in the chemostat can effect the rate of evolution and molecular mode of resistance. For example, the inclusion of a relatively small amount of glass or polystyrene beads, or biological matrix, increases the rate of mutant evolution and selection in the nalixidic acid/*E. coli* ATCC 11775 model system described above. Thus, the present invention is also useful for discovery and characterization of virulence mechanisms and determinants, and can have important applications in the area of biofilms as relevant to virulence.

The foregoing discussion, description, and examples are illustrative of particular embodiments of the invention, but are not meant to be limitations upon the practice thereof. It is the following claims, including all equivalents, which define the scope of the invention.

EXAMPLES

General Methods

General methods in molecular biology: Standard molecular biology techniques known in the art and not specifically described were generally followed as in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York (1989), and in Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley and Sons, Baltimore, Maryland (1989) and in Perbal, *A Practical Guide to Molecular Cloning*, John Wiley & Sons, New York (1988), and in Watson et al., *Recombinant DNA*, Scientific American Books, New York and in Birren et al (eds) *Genome Analysis: A Laboratory Manual* Seies, Vols. 1–4 Cold Spring Harbor Laboratory Press, New York (1998) and methodology as set forth in U.S. Pat. Nos. 4,666,828; 4,683,202; 4,801,531; 5,192,659 and 5,272,057 and incorporated herein by reference. Polymerase chain reaction (PCR) was carried out generally as in *PCR Protocols: A Guide To Methods And Applications*, Academic Press, San Diego, Calif. (1990). In-situ (in-cell) PCR in combination with Flow Cytometry can be used for detection of cells containing specific DNA and mRNA sequences (Testoni et al, 1996, Blood 87:3822.)

Recombinant Protein Purification

Marshak et al, "Strategies for Protein Purification and Characterization. A laboratory course manual." CSHL Press, 1996.

General Methods in Microbiology

Standard microbiology techniques known in the art and not specifically described were generally followed as in Gerhardt et al. (Eds), *Methods for General and Molecular*

Biology, American Society for Microbiology, Washington D.C. (1994), and in Woodford et al. (Eds), *Molecular Bacteriology: Protocols and Clinical Applications*, Humana Press, Totowa, N.J. (1998) and in Demain et al. (Eds), *Manual of Industrial Microbiology and Biotechnology*, American Society for Microbiology, Washington D.C. (1986), and in Brock et al., *Biology of Microorganisms*, 5th Edition, Prentice Hall, New Jersey (1988).

EXAMPLE 1

The present invention has been used to evolve quinolone resistant mutants from the antibiotic sensitive *E. coli* reference strain ATCC 11775 evolved with EMS mutagen in the presence of nalidixic acid as the selective agent, and to correlate the level of resistance with specific point mutations occurring within the quinolone resistance determining region (QRDR) of the gyrase A gene (*gyrA*).

Before proceeding to directed evolution, the survival rate of bacteria upon exposure to different concentrations of mutagens for varying periods of time was determined in growth medium, either LB or TS broth. Bacteria were grown in broth with shaking until they reached log to late log phase. Mutagen was added at this stage and the bacteria were allowed to grow for a specified period of time, depending on the type of mutagen. The mutagenic reaction was terminated by immediately washing and diluting the bacterial cells in suitable buffer or growth medium. The different dilutions of the cells were plated on agar medium to determine the surviving fractions of the cells, which were compared with the non-treated bacterial cell used as control for 100% survival.

The selected concentration of mutagens and the reaction period for *E. coli* ATCC 11775 resulting in an acceptable range of survival rates with reasonable mutational frequencies are shown in Table I.

Proprietary microcarrier spinner flask chemostats were used for continuous culture. Two hundred ml of LB or TS broth containing antifoam A (50 ppm concentration) was autoclaved in the chemostat flask with associated tubes and connectors attached to them. The chemostats were set up, by placing them on a multi-magnetic stirrer, and by connecting them to a constant water supply for providing constant temperature, to a vacuum pump through the waste-disposal reservoir, and to the medium reservoir through a controllable peristaltic pump. The starter culture of bacteria was inoculated into the flask through a sample port by using a 3.0-ml sterile syringe followed by washing the port with an aliquot of sterile broth. The culture was allowed to grow in batch mode with constant stirring until it reached log to late log phase (usually 3.0 to 4.5 hours). At this stage, continuous culture mode was established by feeding the flask with a continuous supply of fresh medium from the reservoir. The flow rate was maintained so that the culture medium could be replaced with the fresh medium during the time period when the culture attains its log phase. OD₆₀₀ was monitored at regular intervals by withdrawing the culture through the sample port with a sterile syringe. The culture was, thus, allowed to maintain several generations at its log phase. Mutagen EMS (2% w/v) or MNNG (10 µg/ml) was added to the continuous culture through one of the infusion ports for a specific period, during which the chemostat was operated in batch mode (30 minutes for EMS and 10 minutes for MNNG). The continuous culture was resumed at a slightly higher flow rate to ensure washing of the mutagen from the chemostat before selection of the culture with the target antibiotic or inhibitor. Alternatively, a low dose or sublethal

concentrations of mutagen (e.g., 0.1–0.5 µg MNNG/ml) was directly added to the medium reservoir in order to maintain the constant exposure of the mutagen in the continuous culture system. Antibiotic was also directly incorporated into the medium reservoir for the selection of mutants from the mutagenized culture after several generations, starting with the sub-lethal concentrations of antibiotic and then with the gradual increase of the antibiotic concentration, depending on the culture growth.

At 8–12 hour intervals a small aliquot of the culture was withdrawn from the chemostat, OD₆₀₀ was measured, and an aliquot of the culture was spread on an agar medium containing a gradient of the selective antibiotic (0–30 µg nalidixic acid/ml).

Antibiotic-resistant clones were selected at varying concentrations along the gradient and further analyzed by a standard antimicrobial disk-diffusion test (antibiogram) for extent of resistance phenotypes (whether partially or completely resistant). Genomic DNA from the selected mutant was prepared and used as template in a PCR designed to amplify the target resistance gene. The PCR product was sequenced directly and analyzed to detect the expected point mutations within the resistance-determining region (QRDR) (Table II). Sequences were routinely aligned and compared with the wild-type (non-mutated) gene or gene region (FIG. 2).

An *E. coli* strain 11775 was also subjected to mutagenesis by EMS in a chemostat continuous culture system and allowed to grow for seven days for over 400 generations. Culture was selected for NA^R mutants on NA-containing gradient plate every 12 hours *E. coli* NA^R clones, thus obtained, from different time-points were analyzed for their phenotype and genetic mutation, especially in the *gyrA* QRDR. Point mutations in the QRDR of several selected NA^R clones from different time-points (generations) are shown in Table III.

The regions of the *gyrA* QRDR sequence of *E. coli* 11775 NA^R mutants, where the mutagen-induced point mutations were consistently observed are shown in FIG. 3. Importantly, controls were conducted in parallel, as described above, and confirmed that the resistant isolates were new mutants and not the result of selection of preexisting mutants.

Most importantly, this example of the present invention accurately mimics both the genotype and phenotype of the clinical resistant isolates (Weigel, L. M., et al., 1998). Nalidixic acid resistant mutants (e.g. NX-E09/11775 and NX-E09/11775) show the specific point mutations observed in several fluoroquinolone resistant clinical isolates (FIG. 2). This occurs in spite of mutagenesis occurring in a random fashion. Thus, the present invention is useful as a means to accurately discover and characterize, through directed evolution, resistance conferring molecular determinants.

EXAMPLE II

An additional demonstration of the method and materials embodying this invention comes from the application of the invention to the evolution of an extended spectrum beta-lactam resistant strain of *Klebsiella pneumoniae*. The development of extended spectrum P-Lactam resistant mutants of *K. pneumoniae* was carried out in similar manner as described in Example I. A clinical isolate, *K. pneumoniae* strain Tim 19, was found to contain the TEM-1 P-lactamase which conferred resistance to ampicillin (AM) and amoxicillin/clavulanate (AMC). However, this strain was sensitive to extended spectrum beta-lactams (ESBLs) such

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as cefotaxime (CTX), ceftazidime (CAZ), cefoxitin (FOX), aztreonam (ATM). This clinical strain was subjected to mutagenesis with MNNG in the continuous culture system for at least eight days under continuous mutagen and antibiotic infusion. ESBL resistant mutants were selected with CTX in the range of 2–550 $\mu\text{g/ml}$. Several CTX^R mutants were obtained and analyzed for their multi-drug- resistance phenotypes (Table IV).

This example again demonstrates the utility of the present invention in providing the methods and materials to discover and characterize antibiotic resistance prior to clinical emergence.

In an embodiment of the present invention, the use of a material that mimics a physiological attachment or colonization site in the chemostat can effect the rate of evolution and molecular mode of resistance. For example, the inclusion of a relatively small amount of glass or polystyrene beads, or biological matrix, increases the rate of mutant evolution and selection in the nalidixic acid/*E. coli* ATCC 11775 model system described above. Thus, the present invention is also useful for discovery and characterization of virulence mechanisms and determinants, and can have important applications in the area of biofilms as relevant to virulence.

An important aspect of the utility of this invention is its ability to predict antibiotic resistance prior to clinical emergence. Prior to the development of the present invention, there had only been a single case where the molecular mechanism of resistance was predicted in vitro prior to this characterization from a clinical isolate (Arlet, G., et al., 1993). Most importantly, the present invention provides

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validated drug intervention targets, useful for screening in the development of next generation compounds. These targets can be either the actual mutant protein target or the resistant strain itself. Validated targets differ from putative or hypothetical targets in that they are proven to confer the observed resistance, and therefore, serve as useful intervention targets for future therapeutic development. Validated targets are provided through the practice of the present invention because directed evolution, mutation, and selection are conducted in vivo, and in the relevant biological host. This differs greatly from any form of in vitro mutagenesis or even evolution conducted in a non-relevant host (nonclinical), and is likely the reason that predicting the molecular mechanism of clinical resistance a priori has only been accomplished once.

Throughout this application, various publications, including United States patents, are referenced by author and year and patents by number. Full citations for the publications are listed below. The disclosures of these publications and patents in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

The invention has been described in an illustrative manner, and it is to be understood that the terminology which has been used is intended to be in the nature of words of description rather than of limitation.

Obviously, many modifications and variations of the present invention are possible in light of the above teachings. It is, therefore, to be understood that within the scope of the appended claims, the invention can be practiced otherwise than as specifically described.

TABLE I

Optimised Parameters of Three Chemical Mutagens for *E. coli*

Mutagen	Concentration	Treatment Period (min)	Survival (%)	Mutant Frequencies*
EMS	2% w/v	30	64	1.2×10^{-7}
NQO	400 $\mu\text{g/ml}$	60	77	0.6×10^{-8}
MNNG	10 $\mu\text{g/ml}$	10	43	1.1×10^{-8}

*Mutants selected for nalidixic acid-resistant (NA^R) phenotype. Data are shown from an average of two experiments. Because of slightly better mutant frequency, EMS and MNNG were used in the subsequent mutagenesis experiments.

TABLE II

Point Mutations in QRDR of *E. coli* by Three Mutagens in Batch Culture

Mutagen	Mutants	Phenotype	Mutation Achieved in QRDR	Mutation Report in Clinical Isolate
EMS	NX-E09	Partially NA ^R	Asp-87 (GAC)→ Ile (ATC)	Asp-87→ Ile
	NX-E10	Completely NA ^R	Ser-83 (TCG)→ Leu (TTG)	Ser-83→ Leu
NQO	NX-N6	Completely NA ^R	Ser-83 (TCG)→ Leu (TTG)	Ser-83→ Leu
	NX-M3	Partially NA ^R	Asp-87 (GAC)→ Gly (GGC)	Asp-87→ Gly
MNNG	NX-M5	Partially NA ^R	Asp-87 (GAC)→ Asn (AAC)	Asp-87→ Asn
	NX-M6	Partially NA ^R	Asp-87 (GAC)→ Gly (GGC)	Asp-87→ Gly

TABLE III

Point Mutations in QRDR of *E. coli* by EMS in Continuous Culture

Mutants	Generations	Phenotype	Mutation Achieved in QRDR	Mutation Report in Clinical Isolate
D7NX-E1	403	Completely NA ^R	Ser-83 (TCG)→ Leu (TTG)	Ser-83→ Leu
D7NX-E2	403	Completely NA ^R	Ser-83 (TCG)→ Leu (TTG)	Ser-83→ Leu

TABLE III-continued

<u>Point Mutations in QRDR of <i>E. coli</i> by EMS in Continuous Culture</u>				
Mutants	Generations	Phenotype	Mutation Achieved in QRDR	Mutation Report in Clinical Isolate
D3NX-E5	135	Completely NA ^R	Ser-83 (TCG)→ Leu (TTG)	Ser-83→ Leu
D3NX-E6	135	Completely NA ^R	Ser-83 (TCG)→ Leu (TTG)	Ser-83→ Leu
D2C2-a	82	Partially NA ^R	Gly-81 (GGT)→ Cys (TGT)	Gly-81→ Cys
D2C2-h	62	Partially NA ^R	Gly-81 (GGT)→ Cys (TGT)	Gly-81→ Cys
D2C2-l	62	Partially NA ^R	Gly-82 (GAC)→ Asn (AAC)	Not Known

TABLE IV

<u>Generation of Multi-Drug-Resistant Mutants of <i>Klebsiella pneumoniae</i> Containing TEM-1 beta-Lactamase by MNNG in Continuous Culture</u>		
TEM-1 b/a-Containing Strain	Drug-Resistance Phenotype	Expected Mutation
WT <i>K. pneumoniae</i> Tim 19	CTX ^S , CAZ ^S , FOX ^S , ATM ^S	None
CTX ^R Mutants (8 analyzed)	CTX ^R , CAZ ^R , FOX ^R , ATM ^R	TEM-1→ TEM-3 (Gln-39→ Lys Glu-104→ Lys Gly-238→ Ser)

^S= Sensitive, ^R= Resistant, CTX = Cefotaxime, CAZ = Cefazidime, FOX = Cefoxitin, ATM = Aztreonam.

References

Dykhuizen, D. E. (1993). "Chemostats used for studying natural selection and adaptive evolution." *Methods Enzymol* 224: 613-31.

Weigel, L. M. C. D. Steward, et al. (1998). "gyrA mutations associated with fluoroquinolone resistance in eight

species of Enterobacteriaceae." *Antimicrob Agents Chemother* 42(10): 2661-7.

Arlet, G., M. Rouveau, et al. (1993). "Novel, plasmid-encoded, TEM-derived extended-spectrum beta-lactamase in *Klebsiella pneumoniae* conferring higher resistance to aztreonam than to extended-spectrum cephalosporins." *Antimicrob Agents Chemother* 37(9): 2020-3.

SEQUENCE LISTING

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What is claimed is:

1. A method of selecting cells resistant to selective agent by inducing directed evolution while applying a selective agent to the cells to determine those cells having resistance.

2. The method according to claim 1, wherein said inducing step includes continuous culturing with the selective agent.

3. The method according to claim 2, wherein said continuous culturing further includes halting the continuous culturing and continuing continuous culturing with the selective agent.

4. The method according to claim 2, wherein said continuous culturing further includes continuously infusing the selective agent.

5. The method according to claim 1, wherein said application step includes culturing the microorganisms in a chemostat.

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6. A method of generating mutant drug targets by inducing directed evolution while applying a selective agent to the cells, thereby determining potential drug targets.

7. The method according to claim 6, wherein said continuous culturing further includes halting the continuous culturing and continuing continuous culturing with the selective agent.

8. The method according to claim 7, wherein said continuous culturing further includes infusing continuously the selective agent.

9. The method according to claim 6, wherein said application step includes culturing the microorganisms in a chemostat.

10. A method of determining emerging resistance by inducing directed evolution while applying a selective agent to cells, thereby selecting those microorganisms having emerging resistance.

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11. The method according to claim **10**, wherein said application step includes continuous culturing with the selective agent.

12. The method according to claim **11**, wherein said continuous culturing further includes halting the continuous culturing and continuing continuous culturing with the selective agent.

13. The method according to claim **11**, wherein said continuous culturing further includes infusing continuously the selective agent.

14. The method according to claim **10**, wherein said application step includes culturing the microorganisms in a chemostat.

15. A method of selecting cells resistant to selective agent by inducing directed evolution while applying a mutagen to the cells to determine those cells having resistance.

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16. The method according to claim **15**, wherein said inducing step includes continuous culturing with the mutagen.

17. The method according to claim **16**, wherein said continuous culturing further includes halting the continuous culturing and continuing continuous culturing with the mutagen.

18. The method according to claim **16**, wherein said continuous culturing further includes continuously infusing the mutagen.

19. The method according to claim **15**, wherein said application step includes culturing the microorganisms in a chemostat.

* * * * *